IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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| In re application of | : | |
| Saiko HOSOKAWA et al. | : | |
| Serial No. 08/450,363 | : | Group Art Unit: 1816 |
| Filed May 25, 1995 | : | Examiner: Ron Schwadron |
| HUMAN MONOCLONAL ANTIBODY SPECIFICALLY BINDING TO SURFACE ANTIGEN OF CANCER CELL MEMBRANE | : | |

DECLARATION

I, Toshiaki Tagawa, a Japanese citizen, resident at A102, 3, Sakuradai, Aoba-ku, Yokohama-shi, Kanagawa-ken, Japan, declare as follows:

1. I graduated from Kyushu University department of chemistry faculty of science in 1982, and finished a master course at said University (enzymology) in 1984.

2. Since April 1984 up to the present time, I have been employed by Mitsubishi Kasei Corporation (now renamed Mitsubishi Chemical Corporation) and engaged in the research and development of protein chemistry and immunoliposomes for targeting chemotherapy against cancer in the Biosciences Laboratory, Research Center of Mitsubishi Kasei Corporation (now renamed Yokohama research center of Mitsubishi Chemical Corporation).

3. My publications include "Targeting efficiency of PEG-immunoliposome-conjugated antibodies at PEG terminals", Advanced Drug Delivery Reviews 24: 235-242, 1997; "Immunoliposomes bearing polyethyleneglycol-coupled Fab' fragment show prolonged circulation time and high extravasation into targeted solid tumors in vivo" FEBS Letters 413: 177-180, 1997; "Improvement of pharmacokinetics and antitumor activity against human hepatoma cell line by using adriamycin-entrapped stealthliposomes" 62: Journal of Surgical Oncology 62: 186-193, 1996; "Improvement of therapeutic effect by using Fab'

fragment in the treatment of carcinoembryonic antigen-positive human tumors with adriamycin-entrapped immunoliposomes" Japanese Journal of Cancer Research 85: 434-440, 1994; "Drug-containing protein-bonded liposome" United States Patent 5,264,221; "Phospholipid derivatized with PEG bifunctional linker and liposome containing it" United States Patent 5,556,948.

4. I am co-inventor of the above-identified patent application. Under my direction and supervision, the following experiment was conducted.

## EXPERIMENT

Preparation of liposomes

A solid lipid mixture of dipalmitoylphosphatidyl choline (DPPC)/cholesterol (Chol)/maleimide-modified dipalmitoylphosphatidyl ethanolamine (DPPE) = 18/10/0.5 was hydrated in 1 ml, per 100 mg of the solid lipid mixture, of a 0.3 M citric acid buffer solution (pH 4.0) by means of a vortex mixer. Then, freezing-thawing was repeated five times to give a multi-lamella liposome. The multi-lamella liposome was filtered under pressure successively through polycarbonate membranes having pore sizes of 200 nm and 100 nm mounted in an extruder (manufactured by Nichiyu Liposome Inc.) while being heated at 60 °C, so as to have a regulated particle size. The resulting liposome solution was neutralized with a 1M NaOH solution, and adriamycin (manufactured by Kyowa Hakko) was added in an amount of 1/10 by weight of the lipid, so that at least 97% of the adriamycin was loaded into the liposome. To the adriamycin-loaded liposome, $F(ab')_2$ of a human monoclonal antibody prepared by using iminothiolane in accordance with the method of Traut et al. (Traut, R.R. et al., Biochemistry 12, 3266 (1973)) was added, and the mixture was reacted to form an antibody-bonded liposome.

Then, a thiol-modified poly(ethylene glycol) (PEG) prepared from 2,4-bis(polyethylene glycol)-6-chloro-s-triazine (manufactured by Seikagaku Kogyo K.K) as described in Section b. of Example 7 of the specification of the present application was added to the antibody-bonded liposome solution obtained above and allowed to react with the

maleimide residues to obtain an antibody-bonded PEG-modified liposome. The unreacted antibody and the unreacted poly(ethylene glycol) were removed by gel filtration with Sepharose CL6B. Liposomes having different degrees of modification by poly(ethylene glycol) were obtained by controlling the time of the reaction of the thiol-modified PEG with the antibody-bonded liposome. The amount of PEG was determined from the intensity of absorption of ultraviolet light at 215 nm after a solution of a liposome solubilized by sodium dodecyl sulfate (SDS) was separated by gel chromatography. The amounts of the bonded poly(ethylene glycol) per 100 mg of the lipid are shown in the following table.

Table

| No. | Amount of PEG mg/100 mg lipid |
|---|---|
| 1 | 0 |
| 2 | 1.01 |
| 3 | 1.72 |
| 4 | 2.69 |
| 5 | 4.43 |
| 6 | 5.78 |
| 7 | 7.80 |

Test on Animals

$1 \times 10^6$ cells of MKN45, a human gastric cancer cell line, were subcutaneously transplanted to thighs of BALB/c nude mise, and after 16 days, the antibody-bonded PEG-modified liposomes having different degrees of modification by PEG obtained above were intravenously administered to the mouse from the tails in an amount of 2 mg/kg as adriamycin. After 24 hours, each mouse was subjected to thoracotomy under pentobarbital and perfused with heparine-containing PBS from the right ventricle to wash away the blood. Then the tumor was extracted.

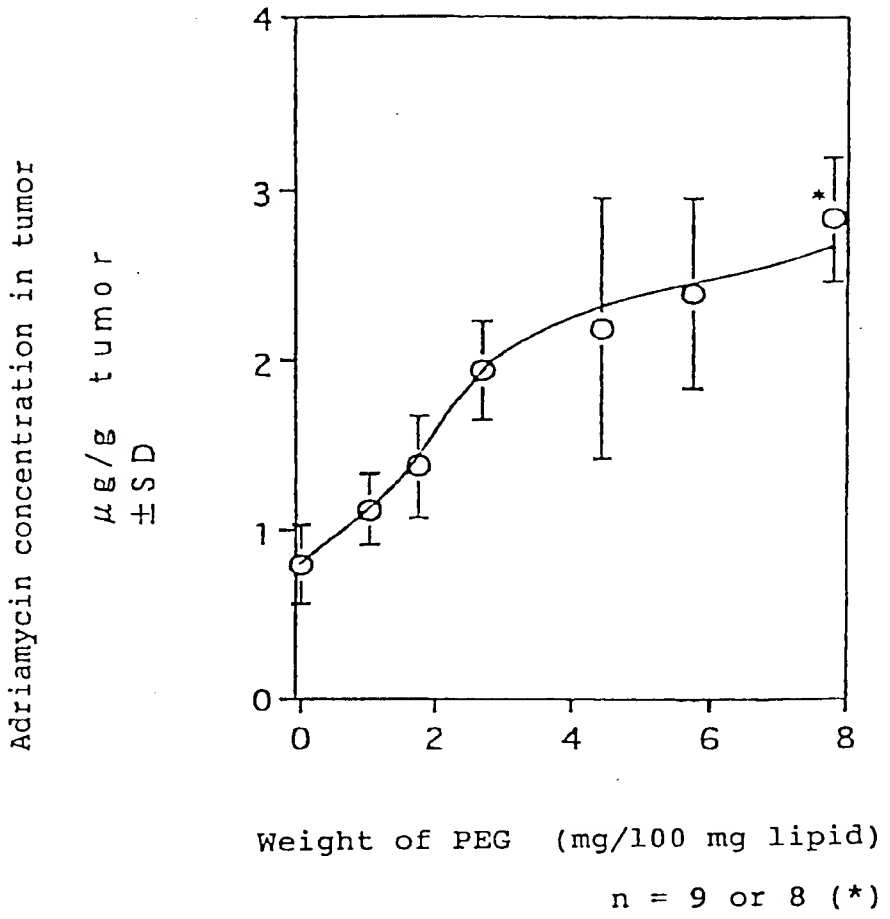
Determination of adriamycin in the tumors

The adriamycin in the tumors was determined by the method of Masuike et al. (Yakugaku Zasshi 104(6) 614 (1984)). A tumor was homogenized in Kolthoff Buffer (pH

8.0) saturated with sodium chloride, and adriamycin was extracted with 4 volumes of chloroform/methanol (4/1). The organic layer was evaporated to dryness, and the residue was dissolved in 50% methanol 0.5 M formic acid. The resulting solution was separated by HPLC at 50 °C by using an ODS column and the same methanol/formic acid as the mobile phase. Adriamycin was determined from the fluorescence of Ex 475 and Em 554 by using daunomycin as the internal standard. The results are shown in the following figure. The abscissa represents the amount of the bonded poly(ethylene glycol) per 100 mg of the lipid, and the ordinate represents the weight of adriamycin accumulated in 1 g of a tumor 24 hours after a liposome was administered in an amount of 2 mg/kg as adriamycin. The data are represented as mean ± SD (N = 9 or 8).

It is evident from the figure that the adriamycin concentration in a tumor increased as the amount of PEG in a liposome increased.

Figure. Relation between the accumulation of adriamycin in tumor and the degree of modification by PEG



I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

January 13, 1998
Date

Toshiaki Tagawa
Toshiaki TAGAWA